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Abstract: INTRODUCTION The mainstays of brain tumor therapy are surgery, radiotherapy and chemotherapy. Cancer immunotherapy is explored as an additional treatment modality. However, emerging evidence indicates that also radio- and chemotherapy have immunological effects in addition to their cytotoxic and cytostatic activities. AREA COVERED We summarize the literature on radio- and chemotherapy-mediated immunological effects in primary and secondary brain tumors and outline open questions within the field. To this end, a literature search was performed using the terms 'brain tumor', 'immune system', 'immunogenic cell death', 'vaccination', 'checkpoint inhibition', 'radiotherapy', 'chemotherapy' and derivations thereof. Expert commentary: Immunological effects of chemo- and radiotherapy in brain tumors involve direct immunogenic modulations of tumor cells, changes of the microenvironment and functional alterations of innate and adaptive immune cells. Each treatment modality can exert various effects that comprise both immune-stimulatory and immunosuppressive mechanisms. A detailed knowledge of these mechanisms is indispensable for an optimal combination of conventional anti-tumor treatments and novel immunotherapeutic approaches.

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Immunological effects of chemotherapy and radiotherapy against brain tumors

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Abstract

Introduction: The mainstays of brain tumor therapy are surgery, radiotherapy and chemotherapy. Cancer immunotherapy is explored as an additional treatment modality. However, emerging evidence indicates that also radio- and chemotherapy have immunological effects in addition to their cytotoxic and cytostatic activities.

Area covered: We summarize the literature on radio- and chemotherapy-mediated immunological effects in primary and secondary brain tumors and outline open questions within the field. To this end, a literature search was performed using the terms “brain tumor”, “immune system”, “immunogenic cell death”, “vaccination”, “checkpoint inhibition”, “radiotherapy”, “chemotherapy” and derivations thereof.

Expert Commentary: Immunological effects of chemo- and radiotherapy in brain tumors involve direct immunogenic modulations of tumor cells, changes of the microenvironment and functional alterations of innate and adaptive immune cells. Each treatment modality can exert various effects that comprise both immune-stimulatory and immunosuppressive mechanisms. A detailed knowledge of these mechanisms is indispensable for an optimal combination of conventional anti-tumor treatments and novel immunotherapeutic approaches.

Keywords: radiotherapy, chemotherapy, immunogenic cell death, vaccination, checkpoint inhibition, brain tumor, glioblastoma

1. Introduction

Brain tumors are a heterogeneous group of intracranial neoplasms representing the most common cancer entity in children and adolescents as well as the seventh most common cancer in adults (1). They are systematically separated in primary brain tumors that originate from cells within the brain or surrounding tissue such as meningeal cells and the more common secondary brain tumors, which represent metastases from extracranial neoplasms. Approximately one third of all primary brain tumors are malignant, which is defined by histopathological features such as increased cellularity, infiltration into the surrounding brain parenchyma and more recently by specific molecular alterations like mutations in the isocitrate dehydrogenase 1 (*IDH1)* gene, the alpha thalassemia/mental retardation syndrome X-linked (*ATRX)* gene or the telomerase reverse transcriptase (*TERT*) promoter (2) in gliomas.

These biological characteristics have therapeutic implications. The vast majority of malignant brain tumors cannot be cured by resection alone. Surgery, however, remains a key element within the management of these neoplasms in order to obtain tissue samples for an accurate histological diagnosis, the determination of molecular markers, as well as the reduction of mass effects which may improve progression-free and overall survival (3, 4). Most treatment regimens against malignant brain tumors comprise radiotherapy (RT) and systemic treatment, either sequentially in time or in combination. For example, the standard of care for patients with newly diagnosed glioblastoma, the most common malignant primary brain tumor in adults, comprises maximal surgical resection followed by temozolomide (TMZ)-based radiochemotherapy (5). Other guideline-based chemotherapeutic protocols for primary brain tumors involve nitrosourea-based regimens such as the combination of

procarbazine, lomustine and vincristine (PCV) (6). Secondary brain tumors are predominantly treated with surgery and/or radiotherapy (7). Chemotherapeutic agents are also active against brain metastases, but are less well studied in randomized trials. Typically, the drugs which act best against the primary tumor are also considered the most active compounds against brain metastases. Hence, different chemotherapy agents are utilized, depending on the histology of the primary tumor. Topoisomerase I (topotecan, irinotecan) and II (etoposide, teniposide) inhibitors as well as platinum-based agents (cisplatin, carboplatin) are used for the treatment of lung cancer brain metastases (8), alkylating drugs such as dacarbazine, TMZ or nitrosoureas are used against melanoma or breast cancer manifestations in the brain (9, 10). Of note, there is no specific chemotherapeutic agent for the treatment of brain metastases.

In addition to these conventional antitumor therapies, immunotherapy is an arising treatment pillar against cancer and various immunotherapeutic approaches are currently being studied against primary and secondary brain tumors (11-13). There is increasing evidence that radio- and chemotherapy may also have immunological effects that can either stimulate or inhibit anti-tumor immune responses. Thus, the combination of conventional treatment modalities with novel immunotherapeutics may result in additional or even synergistic anti-tumor activity but can also lead to deleterious effects. Because of this crucial interaction, we summarize the current knowledge of the immune effects of radio- and chemotherapy against brain tumors. A detailed understanding of these mechanisms could help guiding novel strategies to successfully combine conventional and immunotherapeutic treatment concepts.

2. Chemotherapy

Alkylating chemotherapy is part of the standard treatment regimen against diffusely infiltrating gliomas (14) and also used for therapy of brain metastasis, e.g., from melanoma (15). The addition of TMZ to radiotherapy prolongs survival of patients suffering from glioblastoma or anaplastic glioma without 1p/19q co-deletion (5, 16). The combination of RT and PCV chemotherapy prolongs survival compared to radiotherapy alone in patients with 1p/19q co-deleted anaplastic gliomas and low-grade tumors (17-19). TMZ is orally administered and better tolerated than PCV. Its small size of only 194 daltons and its lipophilic properties enable crossing of the blood-brain barrier (20). A retrospective case control study demonstrated superior efficacy over the functionally similar dacarbazine (DTIC) in preventing isolated central nervous system relapses from metastatic melanoma (21). However, a randomized phase III trial comparing TMZ and dacarbazine in systemically metastatic melanoma demonstrated non-inferiority and did not meet the planned superiority endpoint (22). On a mechanistic level, TMZ methylates guanine in DNA which induces a cycle of DNA mismatch repair that leads to single- and double-strand breaks. This DNA damage triggers autophagy, senescence and apoptosis (23). Interestingly, TMZ may have immune-stimulatory and immune-inhibitory activity beyond its cytostatic and cytotoxic effects. In preclinical glioma and melanoma models, TMZ induced the cell surface exposure of calreticulin (CRT) and the secretion of high-mobility group-1 protein (HMGB1), which rendered tumor cells more susceptible to T cell-mediated killing and phagocytosis (24, 25). CRT, HMGB1 and several other intracellular molecules summarized by Garg at el. (26) can act as danger-associated molecular pattern molecules (DAMP) upon cell surface translocation or extracellular release that stimulate antigen-presenting dendritic cells (DC) and macrophages. If these mechanisms accompany treatment-induced cancer cell death, they can potently

prime adaptive anti-tumor immune responses characterized by target specificity and immunological memory without the requirement of external adjuvants. This immune-stimulatory mechanism of anticancer therapy has been known as immunogenic cell death (27). This TMZ-mediated immune-stimulatory effect has been additionally boosted in combination with gene therapy that promotes the expansion of antigen-presenting cells. This led to long-term survival in murine glioma and intracranial melanoma models (28).

Such immune-stimulatory effects were also observed in clinical trials. Pretreatment of patients within phase I clinical trials with TMZ and RT followed by vaccination with DC pulsed with autologous tumor lysate was safe and increased tumor antigen-specific T cells over the treatment course (29, 30). In a phase II multicenter trial assessing a peptide vaccine targeting the mutant variant III of epidermal growth factor receptor (EGFRvIII), dose-intensified TMZ enhanced humoral and cellular vaccine-induced immune responses despite lymphopenia and enrichment of regulatory T cells (Tregs) (31). The authors of this study emphasized the correct timing of the vaccine relative to the recovery of lymphocytes after TMZ administration, which is a consequence of the hypothetical mechanism underlying this enhanced immune response after treatment-related lymphopenia. It must be assumed that the peripheral lymphocyte pool is reduced by TMZ, which enables thymic-independent antigen-driven T cell proliferation within the context of T cell homeostasis. In addition to this concept of preconditioning with chemotherapy before tumor-specific immunization, the anti-tumor effect of TMZ was also pronounced in the post-vaccination phase in glioblastoma patients treated with autologous peptide-loaded DC (32). It remains unclear whether this was a result of tumor cell sensitization to chemotherapy after vaccination or TMZ-boosted anti-tumor immune responses in the post-vaccination phase.

TMZ may also promote the efficacy of immune checkpoint inhibitors which boost T cell function by counteracting co-inhibitory receptors like cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein 1 (PD-1). The current knowledge from preclinical and clinical studies on immune checkpoint inhibition against primary brain tumors has been summarized by Preusser et al. (33). Brain tumors with high mutational load are particularly responsive to immune checkpoint inhibition (34). TMZ can induce a “hypermutation phenotype” with alterations of DNA mismatch repair (MMR) genes (35, 36). Hence, it needs to be assessed whether brain tumor patients that were treated with TMZ will respond better to immune checkpoint inhibition.

Another mechanism of TMZ that could promote an anti-tumor immune response is the depletion of immune cells with inhibitory properties. TMZ depletes monocytes, which are recruited to the tumor site by chemoattractants and converted to immunosuppressive tumor-associated macrophages and myeloid-suppressor-like cells (37, 38). Furthermore, low-dose TMZ depletes $CD4^+CD25^+Foxp3^+$ Tregs, which are potent suppressors of innate and adaptive immune cells (39, 40).

In contrast to these immune-stimulating effects, TMZ may also dampen immune responses. Fadul et al. assessed the effect of radiotherapy and TMZ on peripheral blood monocytes and lymphocytes in patients with glioblastoma after four weeks of treatment. The authors observed a treatment-related shift towards a suppressive immunophenotype characterized by enrichment of Tregs and decreased NK and $CD8^+$ T cells (41). Another prospective study with a similar design confirmed this treatment-related lymphopenia. However, in this study immunophenotyping revealed that predominantly B and $CD4^+$ T cells were affected, without a significant enrichment of Tregs and stable proportions of NK and $CD8^+$ T cells (42). Thus, a drop in total lymphocyte count following TMZ treatment is a common observation, but

consequences on individual immune cell subpopulations and immunocellular functions remain to be determined in future studies because of the conflicting data. Furthermore, these studies assessed the effect of combined radiochemotherapy without separating the effects of either therapy alone. Results of a retrospective study suggest an association of treatment-related lymphopenia due to radiochemotherapy with reduced overall survival in elderly glioblastoma patients (43).

The potentially inhibiting effects of alkylating chemotherapy on the immune system stimulated research efforts to overcome this problem. One strategy is genetic engineering of immune cells to convey chemoprotection. Maier et al. transduced $CD34^+$ hematopoietic stem cells with lentiviral vectors to overexpress multidrug-resistance-protein 1 (MDR1) and O^6-methylguanine-DNA methyltransferase (MGMT). The transduced cells were resistant to alkylating chemotherapy (44), allowing high-dose treatment with alkylates. Another strategy is the depletion of immune cells with suppressive properties. In the context of brain tumors, this was achieved by administration of a monoclonal antibody against the high affinity interleukin-2 (IL-2) receptor (IL-2Rα/CD25) that is constitutively expressed on Tregs but also transiently on effector T cells. Administration of an anti-CD25 antibody during treatment-related lymphopenia improved the ratio of effector T cells to Tregs and enhanced the effect of anti-tumor immunotherapy (45, 46). However, the authors of these studies emphasize the timing of anti-CD25 application during TMZ-mediated lymphopenia to reduce the likelihood of interference with effector T cell responses.

Other studies suggest that the immunogenic effects of alkylating chemotherapy depend on the type of tumor antigen (47). Non-mutated tumor-associated antigens are recognized by T cell clones that harbor low affinity T cell receptors due to thymic selection processes. These antigens induce a slow proliferative T cell response and the effector cells are additionally controlled by Tregs. In contrast, mutated antigens

are recognized as “non-self” by high-affinity T cell clones and antigen binding results in rapid clonal T cell proliferation that is prone to DNA damage (48). As a consequence, TMZ could boost the immune response to non-mutated tumor-associated antigens through Treg depletion, whereas it potentially inhibits the response to neo-antigens. Indeed, this was demonstrated in model-antigen expressing murine models of melanoma and glioma, in which TMZ abrogated the survival benefit gained with a neo-antigen-based vaccination (49).

The immunological effects of other chemotherapeutic agents that are occasionally used in brain tumors are less well studied and observations made in extracranial neoplasms cannot be simply transferred to the situation in the CNS. Nitrosourea compounds comprise lomustine, which is used alone or in combination with procarbazine and vincristine in patients with malignant gliomas as well as carmustine and fotemustine. The latter is used in metastatic breast cancer and melanoma. A single-arm trial of fotemustine in combination with ipilimumab demonstrated long-term activity against melanoma brain metastases (50, 51), but the putative immunological effects of fotemustine remain elusive. Beside systemic administration, nitrosoureas are occasionally applied in form of local chemotherapy with biodegradable carmustine wafers (52). This treatment, however, is only used at single centers and the immunological effects of this local therapy have not been assessed so far. Platinum compounds such as cisplatin, oxaliplatin and carboplatin may have immunological effects in extracranial cancers through the induction of immunogenic cell death (53) or downregulation of PD-L2 which is a ligand to the inhibiting immune cell receptor PD-1 (54). However, these drugs have only limited, if any, activity against primary brain tumors (55, 56). Irinotecan that belongs to the group of topoisomerase inhibitors facilitated immunosuppression due to accumulation

of myeloid-derived suppressor cells (MDSC) in colorectal cancer (57). Further studies will be needed to understand these mechanisms in more detail.

3. Radiotherapy

Radiotherapy is an essential component in many treatment regimens against primary and metastatic brain tumors either alone or in combination with chemotherapy (6, 58). The classical paradigm for the mechanism of action involves DNA lesions induced by ionizing radiation leading to DNA single- and double-strand beaks and ultimately to cell cycle arrest or cell death. In addition, more recent studies demonstrated DNA damage-independent bystander effects on non-irradiated neighboring cells or abscopal effects, which describe a distant response in tumor lesions outside the irradiation field (59). These effects result from complex interactions of irradiated cells with the surrounding microenvironment and the immune system.

Several immune-stimulating mechanisms of radiation have been demonstrated in the context of brain tumors. Irradiation induces and promotes the release of danger-associated molecular pattern molecules like HMGB1 and heat shock protein 70 (HSP70) from human glioblastoma cells (24, 60). These molecules boost innate immune responses. Furthermore, irradiation may help mounting adaptive immune responses via upregulation of MHC class I molecules, which was accompanied by an increased number of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells in a murine glioblastoma model (61). This effect may enhance the efficacy of tumor-antigen specific vaccination as shown in murine models of glioblastoma and melanoma brain metastases. Only the combination of focal irradiation and a tumor antigen-specific vaccination resulted in strong antitumor immune responses and a significant survival benefit (61, 62). Beside this direct modulation of tumor cell immunogenicity,

irradiation may also induce changes in the microenvironment. Focal brain irradiation induced the expression of chemokine (C-C Motif) ligand 2 (CCL2). In mice, this chemokine attracts the influx of macrophages and the tumor tropism of adoptively transferred T cells to intracranial lymphoma cells (63, 64). However, in gliomas, CCL2 may promote tumor cell invasiveness (65) and recruitment of Treg and MDSC (66). Furthermore, the expression levels of CCL2 in glioblastoma samples from The Cancer Genome Atlas (TCGA) inversely correlated with overall survival and there are efforts aiming at inhibiting the CCL2 chemokine-receptor axis (67).

In glioma patients, a CD8-dominant T cell infiltrate was observed after a combination of gene therapy and radiochemotherapy (68, 69). The study consisted of a single combined treatment arm without control cohorts. Hence, no conclusions regarding the immunogenic effect of each single treatment modality can be drawn. A combined preclinical/phase I study on metastatic melanoma demonstrated a predominant role of irradiation for the diversification of the tumor-reactive T cell receptor repertoire (70). It remains to be determined whether this is the result of tumor antigen induction including mutated neo-antigens following irradiation. The same study demonstrated that irradiation leads to an upregulation of PD-L1 in melanoma cells, which may hamper anti-tumor immunity. This radiation-mediated immunological tolerance mechanism was recently confirmed in liver metastases from colorectal cancer (71). In both studies, blockade of the PD-1 pathway restored anti-tumor immune responses and improved survival emphasizing the rational for a combination of radiation therapy and immune checkpoint inhibition. The combination of local irradiation with immune checkpoint inhibition resulted in a significant survival benefit in preclinical glioma models compared to either treatment alone (72, 73). Other potentially immunosuppressive effects are irradiation-mediated upregulation of TGF-β expression (74) as well as an enrichment of Tregs (75). Furthermore, radiation may

contribute to treatment-related lymphopenia, which occurs in approximately 25-40% of patients with newly diagnosed glioblastoma during treatment (76). Mathematical modeling of standard radiation schemes revealed that during a course of focal radiation for glioblastoma the entire circulating lymphocyte pool receives a potentially lymphotoxic dose (77). However, it has been impossible so far to define the net effect of radiation therapy alone on the lymphocyte count in brain tumor patients, because most patients receive combined radiochemotherapy or concurrent medication like steroids. A rescue strategy consisting of pre-radiation lymphocyte harvesting and reinfusion after completion of radiation therapy did not significantly increase the total lymphocyte count in glioblastoma patients suggesting that a single lymphocyte harvest and reinfusion might not be sufficient to correct treatment related-lymphopenia (78).

4. Concurrent medication in brain tumor management with potential immunological effects

Steroids are often used in the management of brain tumor patients to control tumor-associated edema or nausea and vomiting (79). They have potent immune-inhibitory effects that must be considered in multimodal treatment concepts. Steroids suppress pro-inflammatory cytokines and genes associated with NF-κB signaling resulting in reduced B and T cell function (80). Recent reports demonstrate that steroid administration is an independent predictor of poor outcome in human glioblastoma patients and that steroid treatment could interfere with the efficacy of radio- and chemotherapy in murine glioma models as well as human glioblastoma patients (81, 82). As a consequence, steroids should always be tapered when clinically possible not only because of well-known side effects including myopathy, diabetes or thrombosis, but also due to the reported immunological effects.

Anti-angiogenic therapy could be an alternative to steroids. Bevacizumab, a recombinant humanized monoclonal antibody that inhibits vascular endothelial growth factor (VEGF) is approved by the US Food and Drug Administration as monotherapy treatment for patients with recurrent glioblastoma. VEGF itself has immunosuppressive properties. It inhibits DC maturation (83) and suppresses T cell function (84). In contrast to steroids, anti-VEGF treatment did not interfere with the efficacy of radiotherapy in a murine glioblastoma model (81). In addition, it did not reduce the therapeutic activity of a DC-based vaccine in patients with newly diagnosed glioblastoma (85) and an EGFRvIII-based peptide vaccination in patients with recurrent glioblastoma (86).

5. Expert commentary: authors view on current status of the field

Surgical resection, radiotherapy and chemotherapy are the cornerstones of most treatment regimens against primary and secondary brain tumors. Immunotherapy is a novel treatment option that resulted in improved outcome of patients with various types of extracranial neoplasms. Research efforts aiming at exploring different immunotherapeutic strategies against primary and secondary brain tumors are ongoing. There is also emerging evidence that radio- and chemotherapy significantly impact anti-tumor immune responses. The underlying mechanisms involve direct modulation of tumor cells, changes within the microenvironment and alterations of the immune cell compartment.

Some of these effects and their functional consequences have been characterized in detail such as the immune-stimulatory induction of immunogenic cell death by different chemotherapeutic agents. In contrast, other effects have only been assessed superficially so far like changes in gene or protein expression patterns or alterations of immune cell subpopulations. The functional immunological

consequences of radio- and chemotherapy and the underlying molecular mechanisms that ultimately result in anti-tumor activity have to be explored in more detail in preclinical and translational studies to understand the relevance of these findings. Furthermore, since susceptibility to chemo- or radiotherapy varies among different brain tumor entities, it remains to be clarified if this treatment sensitivity also affects the treatment-associated immunological consequences.

Another topic with increasing importance is immune monitoring at the tumor site. The clinical course of brain tumors is routinely monitored with sequential MRI scans and tumor treatment with chemo- and/or radiotherapy can be accompanied by radiographic effects that mimic tumor progression (87). The pathophysiology and molecular basis for this treatment-related effects are poorly understood but they seem to result from local tissue reaction with inflammation, edema and alteration of vessel permeability (88). A systematic analysis about the contribution of treatment-related inflammatory mechanisms for this phenomenon has not been done. Differentiating “pseudoprogression” from real tumor progression is challenging and efforts are ongoing to incorporate immune-related considerations into imaging assessments (89).

Radio- and chemotherapy may not only confer immune-stimulatory effects but also result in immune-inhibitory activity such as treatment-related lymphopenia that could either promote antigen-driven T cell proliferation or deplete effector T cells. The conditions that maximize the immune-stimulating potential shifting the balance to promote anti-tumor immunity need to be explored in future studies.

Other therapeutic strategies that are currently being investigated aim at counteracting the immunosuppressive effects of radio- and chemotherapy. The emerging class of immune checkpoint inhibitors may be suitable to abrogate the immune-inhibiting effects of conventional treatments such as radiation-mediated induction of PD-L1.

This emphasizes the importance and advantages of combination therapies which need to be explored in carefully designed study concepts. So far, most of the observed immunological effects derived from radio- or chemotherapy stem from preclinical or clinical phase I and II studies which do not allow for final conclusions.

6. Five-year commentary: speculative viewpoint how the field will evolve in 5 years

Traditionally, the anti-tumor effects of radio- and chemotherapy were assessed based on their cytotoxic and cytostatic potential. Today, technological advances allow for a more detailed exploration of these treatment modalities and their immunological effects. High-throughput analyses such as DNA and RNA sequencing as well as proteomics allow for the detection of immune-related gene signatures, mRNA and protein changes in tumor cells and immune cells as well as their modification by radio- and chemotherapy. These approaches might reveal new immune-related molecules that are altered by these conventional treatments. Furthermore, sophisticated immunophenotyping methods like mass cytometry will allow for high-dimensional tracking of the entire immune cell compartment and might translate the molecular modifications to phenotypic changes (90). In addition, methods of computational immunology will evolve and might enable integrative analyses from molecular up to organismal levels.

The identification of immunological effects of conventional treatment modalities beyond their well-known mechanistic action and their functional characterization will trigger the need for predictive markers that may help to guide treatment decisions. Multimodal treatment regimens that combine radio- and chemotherapy with new immunotherapeutic strategies will be tested in detail in the upcoming years.

7. **Key issues:**

- Radiotherapy and chemotherapy are established cornerstones for brain tumor therapy and have not only cytostatic and cytotoxic effects, but also various immunological effects
- Mechanistically, these immune-effects comprise direct immunogenic modulations of tumor cells such as the induction of immunogenic cell death, changes within the microenvironment and alterations of the phenotypic immune cell composition
- The immunological effects of radio- and chemotherapy in brain tumors comprise both immune-stimulating and -inhibitory effects
- Concurrent medication that is frequently used in conjunction with conventional tumor treatment regimens may exert profound immunological effects such as the immunosuppressive effects of corticosteroids
- Strategies that counteract immunosuppressive effects of radio- and chemotherapy are currently investigated including anti-PD-1 approaches to overcome radiation-mediated PD-L1 induction
- Technological advances like gene sequencing, proteomics, or mass cytometry will continuously reveal more immunological effects of conventional therapies
- Future trials which explore radio- and/or chemotherapy against brain tumors should monitor immunological effects and assess multimodal treatment regimens with novel immunotherapeutics

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